Short Communication

Rapid Regeneration of Adventitious Shoots from Tobacco Mesophyll Protoplasts¹⁾

Makiko HAYASHI and Tetsuo NAKAJIMA

Faculty of Agriculture, University of Tokyo, Tokyo 113

A method of making three step cultures for rapid regeneration of adventitious shoots from tobacco protoplasts was established through determining the culture conditions and controlling the growth of callus. By using this method, many adventitious shoots could be obtained from very small calli in only a month.

KEY WORDS : *Nicotiana tabacum*, rapid regeneration, mesophyll protoplast, adventitious shoot

Introduction

In order to apply genetic manipulation using protoplasts to plant breeding, a shortened callus passage for shoot regeneration is necessary. This assumption comes from the fact the extended duration of callus culture causes undesirable genetic variation in regenerated plants (KELLER *et al.* 1982).

In this paper, the culture methods for an effective and rapid regeneration from tobacco mesophyll protoplasts were developed by determining the culture conditions and controlling the growth of callus.

Materials and Methods

Plant material *Nicotiana tabacum* L. cv. Xanthi (supplied by the Japan Tobacco and Salt Public Corporation) was used for the experiments. The plants were potted in vermiculite and watered daily with a solution of Hyponex (5-10-5) diluted to a strength of 1 : 2,000. They were raised in the phytotron under an irradiance approximately at 10,000 lux with metalhalide lamps (Toshiba Yoko D-400). Daylength was set to be 14 hours with a temperature of 30°C during the day and 25°C during the night. When the plant height came to 50 cm, the most expanded leaf was harvested.

Protoplast isolation After the surface of leaves were sterilized, the lower epidermis was peeled off. Then the leaves were cut into ca. 2×3 cm pieces which were floated with the epidermisless side downwards on the enzyme mixture of following composition ; 0.5% Macerozyme R-10, 1.0% Cellulase Onozuka R-10 and 0.7 M D-mannitol. Isolation was carried out in a dark incubator at 27°C for 1.5 hours.

The isolated protoplasts were separated from the undigested leaf parts by filtration with four layers of cotton cheese cloth. The protoplasts were washed by passing through the following mannitol series ; 0.65→0.60→0.55→0.50→0.45 M mannitol solution contain-

Received December 5, 1983

¹⁾ The experiments were carried out in Tissue Culture Facilities, Center of Environment Regulation System for Biology, Faculty of Agriculture, University of Tokyo.

Fig.1 The effect of cold treatment on uniform growth of calli. Number of cells per callus was counted on about 1,500 calli after 7 day protoplast culture.

ing the NAGATA & TAKEBE's minerals (1971). The washing process was carried out by centrifugation at $50\times g$ for 7 minutes.

Protoplast culture The well refined protoplasts (Fig. 2-A) were further washed with the corresponding culture medium (see below). They were finally plated into 0.7% agar medium in Petri dishes of 35 mm diameter at a cell density of $5\times 10^5\sim 1\times 10^6/\text{ml}$. The dishes were sealed with Parafilm M and were placed upside down in a dark incubator at 27°C .

The culture medium for protoplasts was the same as the NAGATA & TAKEBE's medium (1971) except that the myo-inositol concentration was raised from 100 mg/l to 500 mg/l and the D-mannitol concentration was lowered from 0.7 M to 0.4 M.

Results and Discussion

Induction of uniform calli from protoplasts The first cell division of protoplasts was observed after 4 day culture. When most of the protoplasts had entered into the phase of chloroplast aggregation as a sign of cell division (NAGATA & TAKEBE 1970), on the third day in culture, the dishes were incubated at 4°C for 16 hours. After this cold treatment, the dishes were put back to at 27°C .

The effect of the cold treatment is shown in Fig.1. After 7 day culture (3 days after cold treatment), about 80% of calli in the treated dishes consisted of 5 or 6 cells. The dividing frequency of protoplasts was always over 60%. Thus, a high amount of uniform calli could be obtained by this method.

Growth of calli in the second culture The cell division of the calli continued actively until there were 20 cells in the protoplast culture (Fig. 2-B), but further cell division deteriorated. On the 12th day in protoplast culture, just before the delay of cell division started, the agar medium containing calli was cut into small blocks and the agar blocks were put on the second agar medium which was composed of the MURASHIGE & SKOOG's minerals (1962), 2% sucrose, 100 mg/l myo-inositol, 1 mg/l 6-benzyl-

Fig. 2 Plant regeneration from tobacco mesophyll protoplasts. (A) Tobacco mesophyll protoplasts. Bar : $10\mu\text{m}$. (B) Uniform growth of calli at the 12th day in protoplast culture. Bar : $10\mu\text{m}$. (C) The calli at the 7th day in second culture. Bar : $500\mu\text{m}$. (D) The adventitious shoot formed on protoplast-derived callus. Bar : $250\mu\text{m}$. (E) The numerous adventitious shoots obtained in the third culture. (F) The rooted plantlet.

Fig. 3 Schematic representation of the culture procedure for the rapid induction of adventitious shoots.

aminopurine and 0.7% agar. From the second culture, the calli were illuminated at the intensity of 1,000 lux in 14 hour light and 10 hour dark cycles.

In the second culture, it was assumed that auxin and mannitol in the original agar blocks would diffuse into the new agar medium and their concentrations around calli would become diluted.

The calli on the second medium showed vigorous growth and average number of cells per callus was multiplied fivefold, 20 to 100 cells, in 7 day culture. The diameter of calli became about $400\mu\text{m}$ (Fig. 2-C).

Adventitious shoot induction in the third culture After 7 days in the second culture, the original agar blocks were removed from the second agar medium into liquid medium (see below) in test-tubes. The calli were separated each other and also from agar blocks by gently shaking and were again cultured in the liquid medium which was the same composition of the second medium.

The calli grew to globular appearance and a protrusion of shoot primordium appeared as described by RAVEH and GALUN (1975). The visible shoots with small callus were observed on the 10th day of culture (Fig. 2-D). After 20 day culture, about 60% of the inoculated calli formed adventitious shoots (Fig. 2-E). The adventitious shoots could grow to plantlets with roots by further culture (Fig. 2-F).

The entire culture procedure for the regeneration from protoplasts is schematically shown in Fig.3. Thus, the sequence of three step cultures successfully led to the rapid induction of adventitious shoots in very small callus at a considerably high rate.

Some attempts have been made to shorten the culture period for regeneration from tobacco protoplasts. RAVEH *et al.* (1975) and EVANS *et al.* (1983) succeeded in shortening the shoot regeneration period to 7 weeks and 6~7 weeks respectively. In this study, the period could be further shortened to 4~5 weeks. The method of three step cultures was effective as to shortening of the callus passage and also obtaining a high amount of plantlets. Therefore, this method is of possible application to genetic manipulation using protoplasts in higher plants.

Acknowledgement

The authors wish to express their sincere thanks to Dr. K. YAMAMOTO, Faculty of Agriculture, University of Tokyo, for his valuable suggestions. This research was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Literature Cited

- EVANS, D. A., J. E. BRAVO, S. A. KUT and C. F. FLICK 1983. Genetic behavior of somatic hybrids in the genus *Nicotiana*: *N. otophora*, *N. tabacum* and *N. sylvestris*+*N. tabacum*. Theor. Appl. Genet. 65: 93~101.
- KELLER, W. A., G. SETTERFIELD, G. DOUGLAS, S. GLEDDIE and C. NAKAMURA 1982. Production, characterization and utilization of somatic hybrids of higher plants. p.81~114. In: Application of plant cell and tissue culture to agriculture and industry, TOMES, D. T., B. E. ELLIS, P. M. HARNEY, K. J. KASHA and R. L. PETERSON (eds.), The University of Guelph, Canada.
- MURASHIGE, T. and F. SKOOG 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473~497.
- NAGATA, T. and I. TAKEBE 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92: 301~308.
- and ——— 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. ibid. 99: 12~20.
- RAVEH, D. and E. GALUN 1975. Rapid regeneration of plants from tobacco protoplasts plated at low densities. Z. Pflanzenphysiol. 76: 76~79.

タバコ葉肉プロトプラストからの不定芽誘導期間の短縮化

林 万喜子・中島 哲夫

(東京大学農学部, 東京都)

タバコ葉肉プロトプラストから不定芽を急速かつ効率よく誘導する目的で, 培養操作に改良を加えた。

人工気象室内で厳密に生育管理した, 品種キサンチの展開葉を材料とし, プロトプラストを調整した。Nagata & Takebe (1971) のプレート法を一部改変し (マンニトール 0.4M, イノシトール 500mg/l), 細胞分裂前に低温処理 (4°C, 16 時間) を挿入して, カルス生長の斉一化をはかった (第Ⅰ培養)。分裂速度が遅滞する直前 (培養 12 日目) に, 小カルスを含む培地寒天片を Murashige & Skoog (1962) の寒天培地 (蔗糖 2%, BAP 1mg/l) 上に移植して, カルスの生長促進をはかった (第Ⅱ培養)。約 100 細胞期に達した時期 (7 日目) に, 同じ組成の液体培地に移し, 不定芽形成を誘導した (第Ⅲ培養)。

以上, 3段階の培養によって, プロトプラスト培養開始後わずか4~5週間で, 約60%の小カルスに, 不定芽形成を誘導することが可能となった。